Identification and Characterization of Near-Isogenic Hard and Soft Hexaploid Wheats

Craig F. Morris,* Garrison E. King, Robert E. Allan, and Marco C. Simeone

ABSTRACT

A complete understanding of the physical–chemical mechanism and underlying genetic control of wheat (*Triticum aestivum* L.) endosperm texture will contribute to defining optimal grain utilization while assisting the breeding and development of new cultivars. World trade in wheat grain primarily is based on the two main market classes, "soft" and "hard," which are mostly determined by the expression of the puroindoline genes at the *Hardness* (*Ha*) locus. Here we identify and characterize new genetic stocks (near isogenic lines, NILs) in four different genetic backgrounds (20 NILs total, nine hard and 11 soft). Methods included identifying homogenous or mixed texture lines by Single Kernel Characterization System and Near-Infrared Reflectance Spectroscopy. Puroindoline genes and *Ha* alleles were determined through nucleic acid sequence analysis. The four different genetic sources for NILs were (i) accessions of 'Gamenya' cultivar which were physical mixtures of hard and soft types, (ii) existing near-isogenic lines from the cultivars Heron and Falcon, (iii) advanced-generation backcross lines involving 'Paha' and 'Early Blackhull,' and (iv) 'Nugaines' and 'Early Blackhull Derivative'. The NILs reported here provide new genetic materials for the study of wheat grain texture and the effect of puroindolines and the *Hardness* gene on end-use quality. Two of the four sets of NILs possess the Gly-46 to Ser-46 *Pinb-D1b* hardness allele which has not been previously available in NILs. The results corroborate a model of wheat grain texture that identifies two major hardness classes, as opposed to one that accommodates intermediate texture classes such as "semi-hard" and "medium-soft." A direct role of the puroindoline proteins in conferring soft grain phenotype is supported; conversely, no genetic basis for intermediate hardness was found. Rather, intermediate hardness resulted from mixtures of the soft and hard classes.

KERNEL HARDNESS (texture) is arguably the single most important determinant of wheat grain quality and utilization (Pomeranz and Williams, 1990; Morris, 1992; Morris and Rose, 1996; Campbell et al., 1999). The majority of phenotypic variation among cultivars and individual grain lots for kernel texture can be attributed to the *Ha* gene, located on the short arm of chromosome 5D (Mattern et al., 1973; Law et al., 1978; Symes, 1965; Baker, 1977). Defined genetic stocks that differ in either the presence or absence of the hardness gene, or differ in their allelic state (*Ha*, soft vs. *ha*, hard) are valuable in studying the biochemical and genetic bases of wheat grain hardness and provide material to compare the effect(s) of this gene on wheat and flour processing and end-use quality (Symes, 1969; Simmonds, 1974; Barlow et al., 1973; Rogers et al., 1993; Giroux and Morris, 1997, 1998; Bergman et al., 1998; Morris et al., 1999).

Regarding the underlying molecular basis of grain hardness, Greenwell and Schofield (1986) reported that the presence of the starch surface protein friabilin was associated with grain softness. The occurrence of friabilin was inherited additively (Bettge et al., 1995) and mediated by bound polar lipids (Greenblatt et al., 1995). Friabilin proved to be identical to the Triton X-114 soluble proteins, puroindolines a and b (Blochet et al., 1993; Jolly et al., 1993; Morris et al., 1994; Gautier et al., 1994). Recent studies revealed that as many as four specific mutations in the puroindoline genes confer wheat grain hardness (as opposed to soft which is the wild-type allele). The first mutation involves a complete absence of puroindoline a mRNA and protein (Giroux and Morris, 1998), whereas the second involves a single nucleotide change in puroindoline b such that the Gly-46 residue of the protein is altered to a Ser-46 (Giroux and Morris, 1997). The third and fourth mutations are also single-nucleotide changes such that Leu-60 is changed to Pro-60, and Trp-44 is changed to Arg-44, respectively (Lillemo and Morris, 2000). To date, a survey of several hundred hard wheats indicates that all *ha* (hard) alleles are attributable to specific changes in these puroindoline proteins.

An issue related to wheat grain hardness, utilization, and the breeding of improved cultivars is the existence of what is commonly referred to as "semi-soft" or "semi-hard" wheats. We have been keen to better understand and elucidate the genetic basis for such wheats as they do not apparently fit the current genetic model of hardness described above. One such semi-soft cultivar is Gamenya (Crosbie, 1989). A second example of possible semi-soft genotypes occurred among the set of 'Falcon' and 'Heron' hard and soft NILs (Symes, 1965; Giroux and Morris, 1998). However, the two lines that exhibited intermediate levels of grain hardness were found to be mixtures of hard and soft types (Giroux and Morris, 1998). To date, the set of Falcon and Heron NILs has been one of the primary genetic materials for studying the effects of *Ha* (Rogers et al., 1993). The recombinant inbred lines derived from 'Clark's Cream' and 'NY6432-18' represent another (Campbell et al., 1999). Campbell et al. (1999) showed how the hardness gene was an often overwhelming contributor to variation in end-use quality among those Clark's Cream X NY6432-18 lines.

During the course of our studies on Gamenya, we found that the semi-soft character of some earlier types of this cultivar appear to have resulted from mixtures of hard and soft types. Sibling lines have been isolated

C.F. Morris and R.E. Allan (retired), USDA-ARS, Pullman, WA 99164; G.E. King, Dep. Food Sci. & Human Nutrition and M.C. Simeone, Dep. Crop & Soil Sciences, Washington St. Univ., Pullman, WA 99164.
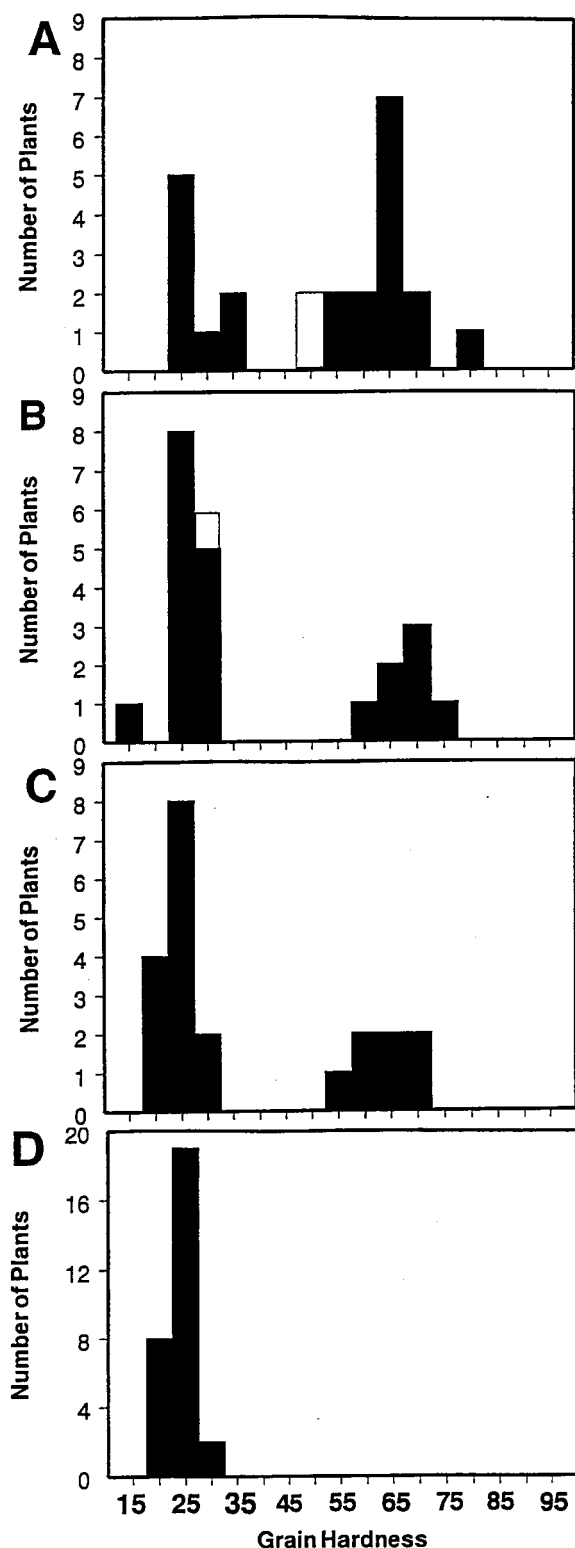
Fig. 1. Frequency distribution of Single Kernel Characterization System (SKCS) grain hardness of individual plants of four accessions of the Australian cultivar Gamenya obtained from the USDA NSGC. (A) PI 268329, (B) PI 274503, (C) PI 290909, and (D) PI 349638. Filled bars represent individual plants used to construct hard and soft NILs; open bars represent individual plants with higher SKCS standard deviations and therefore considered possibly heterogeneous and were not included in the NILs. PI 349638 was uniformly soft and therefore did not generate a hard NIL.

and characterized. Similarly, the heterogeneous Falcon and Herron lines have been reselected for homogeneity of hardness alleles, and characterized. Lastly, we have identified and researched two additional genetic backgrounds that provided the opportunity of recovering near-isogenic hard and soft lines. Hard and soft NILs from those two new genetic backgrounds have been isolated and characterized. These stocks will provide valuable resources for the study of wheat grain hardness and quality and have been released by the USDA-ARS, registered with the Crop Science Society of America (Morris and Allan, 2001), and deposited in the USDA-NSGC.

MATERIALS AND METHODS

Set 1. Gamenya Sibs

There were four germplasm accessions of the Australian cultivar Gamenya (PI 268329, PI 274503, PI 290909, and PI 349638) in the USDA National Small Grains Collection (NSGC) at Aberdeen, ID. Seed of each was obtained and increased through several cycles of seed amplification and grown in unreplicated field plots (approximately 1.2 by 20 m) at the Spillman Farm, Washington State Univ., Pullman, WA (annual crop) and the Dryland Research Unit, Washington State Univ., Lind, WA (summer fallow) in 1995 under common cultural practices. Grain was harvested and evaluated for grain hardness by near-infrared reflectance spectroscopy (NIR) (model 450, Technicon, Tarrytown, NY) (Method 39-70A; AACC, 1995) and a commercial prototype (P3) of the Single Kernel Characterization System (SKCS) (SKCS 4100, Perten Instruments, Springfield, IL) (Martin et al., 1993) (see Table 1).

New germplasm of those four Gamenya accessions was obtained from the NSGC and used to hand-sow directly 21 to 29 individual plants of each accession at the Western Wheat Quality Laboratory (WWQL) Barmore Farm, Pullman, WA, in 1996 under highly amended soil, irrigation, and manual culture techniques. Individual plants were harvested at maturity, threshed, and grain hardness was determined as described above on 45 to 200 individual kernels from each plant by the SKCS. For each accession, remnant grain of plants with SKCS hardness standard deviations from 12 to 19 was bulked according to soft or hard class (see Fig. 1). Grain from three plants with SKCS hardness standard deviations of 21 to 29 were not included (open bars, Fig. 1). These newly created hard and soft composites for each of the accessions PI 268329, PI 274503, and PI 290909 (PI 349638 was uniformly soft; see Table 1, Fig. 1, and *Results*) were increased near Brawley, CA, during the winter of 1997-1998 by Western Agricultural Research and Production, Inc., Pullman, WA, in a nonrandomized two-replicate design under common cultural practices. Grain from this increase was subjected to a final evaluation

Table 1. Grain hardness of four accessions of Gamenya wheat cultivar grown at two Washington locations in 1995 as determined by near-infrared reflectance spectroscopy (NIR) and the Single Kernel Characterization System (SKCS)†.

	Lind		Pullman	
Accession	NIR	SKCS	NIR	SKCS
PI268329	42	55 ± 26	42	62 ± 22
PI274503	24	50 ± 28	41	58 ± 23
PI290909	23	39 ± 23	24	50 ± 20
PI349638	23	33 ± 19	16	47 ± 16

† ANOVA on the combined accession—location data set produced an LSD ($P = 0.05$) of 23 for NIR and 7 for SKCS for comparison of accession means across locations.

Table 2. Grain hardness of hard and soft near-isogenic line (NIL) composites derived from three accessions of Gamenya wheat cultivar and grown near Brawley, CA, 1997-1998 as determined by near-infrared reflectance spectroscopy (NIR) and the Single Kernel Characterization System (SKCS).

|	Soft NIL			Hard NIL		
Accession	NIR	SKCS	N†	NIR	SKCS	N†
PI268329	31	39 ± 14	13	89	68 ± 13	7
PI274503	36	36 ± 14	8	93	70 ± 16	12
PI290909	27	39 ± 15	14	85	65 ± 15	7

† Number of plants used to create the particular composite seed stock for that NIL.

of grain hardness using the above procedures (see Table 2) and represents the hard and soft near-isogenic sib lines (NILs) described herein. Germplasm of these lines was deposited with the USDA-NSGC (Morris and Allan, 2001).

Set 2. Australian Falcon–Heron NILs

Germplasm of 44 accessions of hard and soft NILs in the Falcon and Heron cultivar backgrounds was obtained from Dr. Michael Mackay, Australian Cereals Collection, Tamworth, NSW. Their AUS numbers are 10751, 10752, 90077, 90252-90261, 90264-90283, and 90286-90296. According to Dr. Mackay (1996, personal communication), accessions AUS10751, AUS10752, and AUS90077 were deposited by Dr. Albert Pugsley, whereas the remainder were deposited by Dr. K.J. Symes. Twenty-five lines had the pedigree Heron/7*Falcon and 19 had Falcon/7*Heron. All 44 lines were grown at Pullman, WA, in 1994 and at Lind, WA, in 1995 with the cooperation of Mr. Mike Davis. Grain of each line at each location was analyzed for grain hardness by the SKCS 4100 using 300 kernels as described above (see Fig. 2).

AUS90077 and AUS90254 were seeded at Barmore Farm in 1997 in unreplicated rows and individual spikes were harvested, threshed and 10 to 12 kernels from each were subjected to SKCS 4100 grain hardness measurement. From the distributions presented in Fig. 3, remnant grain from 10 to 20 spikes from each accession–hardness class combination was bulked and increased near Brawley, CA, as described for Gamenya. Grain harvested from those plots was evaluated for grain hardness using the NIR and SKCS 4100 procedures (see Table
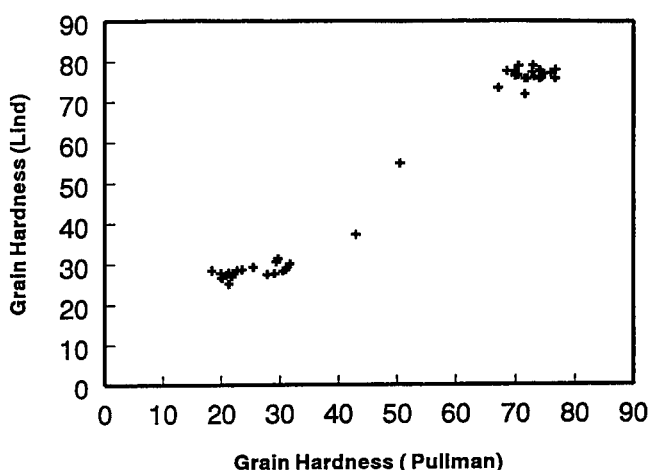
Fig. 2. Single Kernel Characterization System (SKCS) grain hardness of 44 hard and soft NILs derived from Falcon and Heron wheat cultivars, and grown at Pullman (1994) and Lind (1995), Washington. Two of the lines were heterogeneous for hardness whereas the others were uniformly soft or hard.
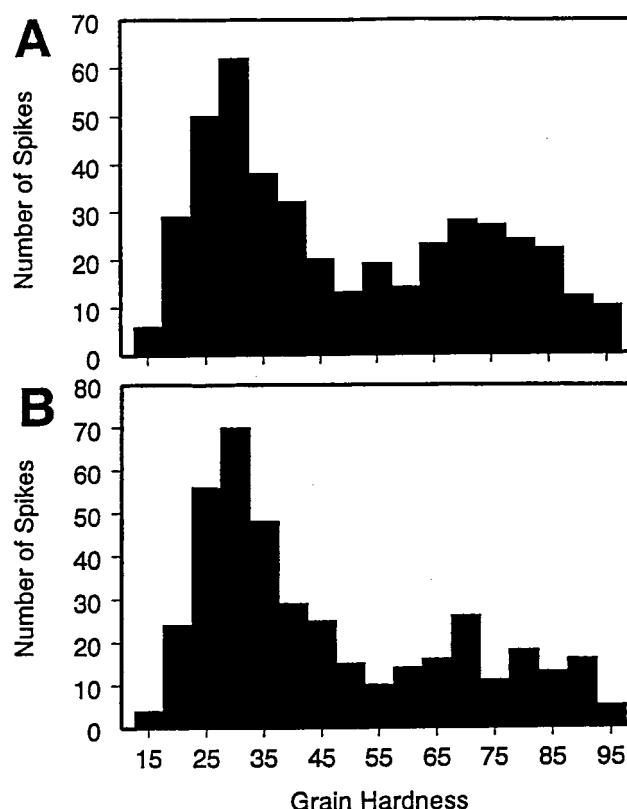
Fig. 3. Frequency distribution of Single Kernel Characterization System (SKCS) grain hardness of individual spikes of two hard and soft NILs derived from Falcon and Heron wheat cultivars that were heterogeneous for grain hardness (see Fig. 2). (A) AUS90077, (B) AUS90254.

3). Germplasm of these four lines was deposited with the USDA NSGC (Morris and Allan, 2001).

To supplement the original set of 44 lines, the following additional accessions were obtained from Dr. Mackay (with supplemental identification): AUS90076 (Pugsley line), 90262 (Heron parent), 90263 (Falcon parent), 90284 (Symes Heron parent), and 90285 (Symes Falcon parent). Accessions, two pots of each, three plants per pot, were increased in a glasshouse during the winter of 1997-1998. Spikes were harvested, threshed, and the grain analyzed for SKCS hardness as above.

Set 3. Paha–Early Blackhull NILs

Twenty-eight $BC_6 F_3$-derived lines of the pedigree 'Paha'*2//'Early Blackhull'/5*Paha, developed primarily for club and lax head type NIL purposes, and the Paha parent were grown at Spillman Farm, Washington State Univ., Pullman, WA (two planting dates) and Central Ferry, WA, in 1994-1995 and 1995-1996 in unreplicated field plots. Early Blackhull was grown

Table 3. Grain hardness of hard and soft near-isogenic line (NIL) composites derived from two heterogeneous accessions of Falcon and Heron wheat cultivar NILs and grown near Brawley, CA, in 1997-1998 as determined by near-infrared reflectance spectroscopy (NIR) and the Single Kernel Characterization System (SKCS).

	Soft NIL		Hard NIL	
Accession	NIR	SKCS	NIR	SKCS
AUS 90077	41	37 ± 14	81	69 ± 16
AUS 90254	38	35 ± 14	93	69 ± 13

Table 4. Grain hardness of hard and soft club-headed near-iso-genic lines (NILs) derived from Early Blackhull and Paha wheat cultivars, and grown at three Washington environments each in 1994-1995 and 1995-1996, as determined by near-infra-red reflectance spectroscopy (NIR) and the Single Kernel Characterization System (SKCS).

Group† or parent	N‡	NIR hardness		SKCS hardness	
		Mean	Range	Mean	Range
Hard NILs	4	67	63-68	68	68-69
Soft NILs	24	22	17-24	34	26-37
Paha	–	22	–	36	–
Early Blackhull§	–	46	–	65	–

† Each hard or soft NIL group was comprised of lines that did not differ significantly for hardness as determined by Duncan's Multiple Range Test [LSD ($P = 0.05$) = 7 for NIR and 6 for SKCS].
‡ N equals the number of lines in that particular NIL group.
§ Early Blackhull was grown in 1996 only ($n = 3$).

in 1995-1996 only. Grain from all six environments was assessed for grain hardness by NIR and SKCS as described above (see Table 4). From these 28 lines, six were chosen on the basis of kernel texture, grain color, relative maturity and plant height, the specific parental plants crossed, and club and awnless spike morphology. Grain of the selected lines was hand harvested from plots grown at Spillman Farm in 1995-1996 and used as seed stock for the hard and soft NILs described herein (see Table 5). Germplasm of these lines was deposited with the USDA-NSGC (Morris and Allan, 2001).

Set 4. Nugaines–Early Blackhull Derivative NILs

Four lines (designated V60, V68, V75, and V112; Table 6) were originally chosen from a larger set of 12 BC$_4$ F$_3$-derived lines based on NIR grain hardness data (Haro, 1990). The set of 12 lines included six with the pedigree, Early Blackhull Derivative/5*Nugaines and six with the pedigree, Early Blackhull/5*Nugaines. All four of the selected lines came from the first pedigree as the lines with the second pedigree were all soft. Those lines were originally developed to be near-isogenic for heading date (Haro, 1990; Haro and Allan, 1997). Haro (1990) grew the lines at the Spillman Farm and near Walla Walla, WA, in 1986-1987 and in 1987-1988 (with two planting dates at Spillman Farm in 1987-1988) for a total of five environments (see Table 6). Reselections of the lines were made in 1992 to verify their uniformity for heading date.

From two to five F$_7$-derived reselections from each of the original lines were grown again in 1993-1994 and 1995-1996 at the Spillman Farm, Pullman, WA, and characterized for hardness as described (see Table 6). Final line selection was

Table 5. Grain hardness class as determined by near-infrared reflectance spectroscopy and the Single Kernel Characterization System, grain color, relative maturity, and plant height of six selected club-headed hard–soft NILs derived from Paha and Early Blackhull wheat cultivars, and parents.

NIL or parent	Hardness class†	Grain color	Relative maturity‡	Plant height§
				cm
655	Hard	Red	+7	95
656	Hard	White	+7	94
678	Soft	White	+7	102
651	Soft	White	+1	83
653	Soft	Red	+1	86
661	Soft	White	+1	98
Paha	Soft	White	+8	101
Early Blackhull	Hard	Red	0	109

† Hardness class (group) data ranges are shown in Table 4.
‡ Maturity relative to Early Blackhull, means over seven environments.
§ Plant height means over seven environments.

Table 6. Grain hardness of hard and soft NILs derived from Early Blackhull Derivative and Nugaines wheat cultivars, as determined by near-infrared reflectance spectroscopy (NIR) and the Single Kernel Characterization System (SKCS).

Line or parent†	NIR hardness‡	NIR hardness§	SKCS hardness§
V60, 1142	76 ± 4	59 ± 4	62 ± 4
V68, 1145	43 ± 2	17 ± 1	31 ± 3
V75, 1153	69 ± 2	46 ± 12	56 ± 7
V112, 1166	39 ± 1	2 ± 13	31 ± 7
Early Blackhull Derivative	55 ± 4	29 ± 13	30 ± 18
Nugaines	44 ± 6	17 ± 6	31 ± 1

† Line designations follow those of Haro (1990).
‡ Data derived from Haro (1990); mean of five Washington environments, two from 1986/87 and three from 1987/88. BC$_4$ F$_3$-derived lines.
§ Mean of two Washington environments (Pullman, 1993/94 and 1995/96). BC$_4$ F$_7$-derived re-selected lines.

based on kernel texture, relative heading date, and plant height (Morris and Allan, 2001).

Statistical analyses were conducted by SAS procedures (SAS, 1989). NIR and SKCS hardness scores are dimensionless and as such, have no units associated with them. DNA isolation from individual kernels, PCR and nucleic acid sequencing procedures were conducted as described by Lillemo and Morris (2000).

RESULTS AND DISCUSSION
Set 1. Gamenya Sibs

Australian Standard White (ASW) wheat class from Western Australia was recognized in the late 1970s as having superior quality for Japanese white salted noodles (Nagao et al., 1977; Oda et al., 1980). At that time, Gamenya was the prominent cultivar of that region. Also, ASW at that time was a class that received both hard and soft-grain cultivars (Crosbie, 1989). As such, ASW was distinct from Australian soft (AS) and the hard classes, Prime Hard and Australian Hard. During the early 1980s, the WWQL received anecdotal information via U.S. wheat marketers that a desirable trait of noodle wheat cultivars was "medium" grain hardness. To learn more about the quality requirements of Asian noodle wheat cultivars, we obtained the four existing accessions of Gamenya from the USDA-NSGC for testing and evaluation. The preliminary results obtained on large field plots (after several generations of seed amplification) are presented in Table 1. Clearly, two features of the material became apparent. First, the accessions differed in grain hardness with PI349683 being the softest and PI268329 being the hardest, and second, grain hardness values and SKCS hardness standard deviations of all but PI349638 were indicative of physical mixtures of hard and soft kernels. The two traditional hardness classes of U.S. wheats are centered on about 25 for soft wheats and 75 for hard wheats (Norris et al., 1989). A major limitation with NIR grain hardness (as well as particle size index, PSI, Williams, 1979; Symes, 1965) has been that they are necessarily conducted on bulk, ground samples and therefore cannot provide information on mixtures beyond the general effect on the value obtained which may be intermediate between the traditional classes. With the development of the Single

Kernel Characterization System (SKCS) (Martin et al., 1993), this limitation was overcome. The SKCS crushes a population of individual kernels and produces a mean and standard deviation grain hardness values. Empirically, we at the WWQL have learned that an SKCS standard deviation on 300 kernels of approximately 13 to 17 is common and indicates an homogeneous sample—hard or soft, whereas a standard deviation of about 20 or greater is generally indicative that the grain lot is mixed. Again, the SKCS hardness value standard deviations of PI268329, PI274503, and PI290909 indicated that they were likely heterogeneous for grain hardness. Those results are consistent with our research on grain hardness, which indicates that medium hardness values of advanced generation, homozygous material are not consistent with the major effects and allelic states of the *Ha* gene and therefore have no genetic basis as such (Giroux and Morris, 1997, 1998; Morris et al., 1999; Lillemo and Morris, 2000; cf. Bettge et al., 1995). Consequently, we investigated whether one or more of these Gamenya cultivar accessions was indeed a mixture of hard and soft grain types, and if so, to separate the genotypes.

To address this issue, individual plants were grown of each accession and analyzed for grain hardness. Grain hardness for each of the individual plants of each of the four Gamenya accessions are presented in Fig. 1. PI268329, PI274503, and PI290909 exhibited two distinct, nonoverlapping populations of grain hardness characteristic of normal soft and hard wheats. PI349638 exhibited homogeneous, soft grain, consistent with prior observation (Table 1). On the basis of those data, hard and soft bulks from each of the three heterogeneous accessions were created by bulking remnant grain of appropriate plants. Three plants exhibiting more medium-tending hardness and high standard deviations were suspect and were excluded from the bulks. They represent about 5% of the total 64 plants examined for these three accessions (Fig. 1 and Table 2). Analysis of the progeny of these bulks confirmed the separation of the soft and hard allelic types (Table 2). PCR analysis indicated that the hard component of these lines carried the *Pina-D1b* hardness mutation ("puroindoline a null") (Giroux and Morris, 1998). Germplasm of these lines has been released by the USDA-ARS and deposited with the USDA-NSGC (Morris and Allan, 2001).

Apparently Gamenya was considered to be a semisoft or medium hardness wheat cultivar on the basis of bulk analysis of grain hardness, traditionally PSI, or particle size index (Crosbie, 1989). From our results, it is clear that it was simply a mixture of soft and hard types. PI349638 appears to represent the "current" Gamenya cultivar since it is uniformly soft, consistent with current production of this cultivar in Western Australia (G. Crosbie, 1997, personal communication).

Set 2. Australian Falcon–Heron NILs

SKCS grain hardness values of the 44 Falcon and Heron soft–hard NILs from two separate locations and crop years are plotted in Fig. 2. Clearly, all but two lines clustered tightly in either the soft or hard grain hardness groups. Two lines, however, were intermediate between the two classes and differed significantly. ANOVA on the combined location hardness data produced an LSD ($P = 0.05$) of 5.2. The two intermediate lines, AUS9077 and AUS90254, were selected by Giroux and Morris (1998) for further study as to the molecular genetic basis of their intermediate phenotype. On the basis of individual kernels of these accessions, Giroux and Morris (1998) showed that they, too, were physical mixtures of hard and soft allelic types, in this case puroindoline a nulls (*Pina-D1b*) and soft, wild type (*Pina-D1a*), respectively.

To separate these two allelic types, a large number of individual spikes of the heterogeneous accessions, AUS90077 and AUS90254, were harvested, threshed, and a few kernels from each analyzed by SKCS 4100. A plot of the resulting data confirmed their mixed nature (Fig. 3). On the basis of these data, once again, hard and soft bulks were created for each accession and used as seed stock for increase. NIR and SKCS grain hardness analysis on the progeny of these bulks confirmed the separation of the two allelic types (Table 3). Germplasm of these lines has been released by the USDA-ARS and deposited with the USDA-NSGC (Morris and Allan, 2001).

For completeness, additional Falcon and Heron lines not included in the original set, but available from the Australian collection were obtained, increased, and evaluated for SKCS grain hardness. Results were as follows: AUS90076, 53 ± 14, 90262, 17 ± 11, 90263, 65 ± 11, 90284, 4 ± 13, and 90285, 49 ± 26. These results are consistent with expectations with one exception, that AUS90285 appears to be heterogeneous for grain hardness, like the two lines AUS90077 and 90254. The heterogeneous nature of the accession would not be consistent with its classification as "Symes' Falcon parent."

Set 3. Paha–Early Blackhull NILs

We serendipitously encountered both soft and hard lines in advanced-generation backcross programs aimed at producing NILs for other traits. The first of these programs involved club (*C*) versus lax (*c*) spike type using the cultivars Paha (club and soft) and Early Blackhull (lax and hard). Results of six environment-years of hardness data are presented in Table 4 and highlight the allelic difference for hardness among these lines. All the lines had low SKCS grain hardness standard deviations and appeared uniformly hard or soft (data not shown). From the set of 28 lines, we chose two hard and four soft lines on the basis of kernel texture, grain color, relative maturity and plant height (Table 5), and the specific parental plants crossed (data not shown). All are winter habit with awnless, red-chaff, club-type spikes.

PCR analysis and puroindoline gene sequencing indicated that the hard lines carry the Gly-46 to Ser-46 *Pinb-D1b* hardness mutation from Early Blackhull (Table 7). This hardness allele most certainly traces its origin to

Table 7. Selected puroindoline gene sequence of Early Blackhull, Paha, hard and soft NILs derived from the cross, Paha*2//Early Blackhull/5*Paha, Early Blackhull Derivative, Nugaines, and hard and soft NILs derived from the cross, Early Blackhull Derivative/5*Nugaines.

	Amino acid position										
	39	40	41	42	43	44	45	46	47	48	49
Early Blackhull, Early Blackhull Derivative, and hard NILs derived from the two crosses	TGG W	CCC P	ACA T	AAA K	TGG W	TGG W	AAG K	AGC S	GGC G	TGT C	GAG E
Paha, Nugaines, and soft NILs derived from the two crosses	TGG W	CCC P	ACA T	AAA K	TGG W	TGG W	AAG K	GGC G	GGC G	TGT C	GAG E

introductions of 'Turkey Red' (Bayles and Clark, 1954). Germplasm of these lines has been released by the USDA-ARS and deposited with the USDA-NSGC (Morris and Allan, 2001).

Set 4. Nugaines–Early Blackhull Derivative NILs

The second set of hard-soft NILs was encountered in a set of "heading-date" NILs derived from the soft white winter cultivar Nugaines and Early Blackhull Derivative, a hard red winter heading date variant of Early Blackhull (Haro, 1990). The original observation of variation in kernel texture was made on the BC$_4$ F$_3$-derived lines described by Haro (1990) and Haro and Allan (1997). The 2 to 5 F$_7$-derived reselections within each of the original lines were all uniformly soft or hard, consistent with the hardness class of the original line (data not shown). Consequently, one each of the reselected lines was chosen to be advanced as parental stock for the hard–soft NILs (Table 6). Final line selection was based on kernel texture, relative heading date, and plant height (Morris and Allan, 2001).

PCR analysis and puroindoline gene sequencing confirmed that these hard NILs also carry the Gly-46 to Ser-46 *Pinb-D1b* allele like Early Blackhull and Early Blackhull Derivative (Table 7). Germplasm of these lines has been released by the USDA-ARS and deposited with the USDA-NSGC (Morris and Allan, 2001).

CONCLUSION

The results presented here support the model that the two major alleles (*Ha* and *ha*) at the *Hardness* locus confer the major variation in grain hardness between hardness classes. Further, prior situations involving "medium hardness" Gamenya cultivar and Falcon–Heron hard–soft NILs proved to be simply physical mixtures of the two types of alleles. Consequently, the genetic basis, if any, to support "medium hard" or "medium soft" wheats remains to be substantiated in other materials.

An elaboration of the hardness gene model which involves the expression of puroindoline proteins was supported without exception by the present study. Giroux and Morris (1997, 1998) and Lillemo and Morris (2000) described specific mutations in the puroindoline proteins a and b that confer hard kernel texture. The first two and most prevalent of these hardness mutations, *Pina-D1b* and *Pinb-D1b*, were both encountered in the present study. Gamenya carries the puroindoline a null mutation (*Pina-D1b*), whereas the hard lines derived from Early Blackhull and Early Blackhull Derivative carry the *Pinb-D1b* allele. This latter allele putatively originated with Turkey Red introductions to Kansas in the latter half of the 19th century. A survey of historically important hard red winter wheat cultivars of North America indicates that greater than 95% carry this hardness mutation (Morris et al., 2001).

These NILs described herein should prove useful in studies aimed at understanding the role of the hardness gene on end-use quality. In addition to adding three new genetic backgrounds to the existing set of NILs derived from Falcon and Heron, these NILs provide a second hardness mutation, winter and spring-habit lines, and lines with a high degree of genetic similarity to the Pacific Northwest soft white club and common cultivars Paha and Nugaines (respectively).

REFERENCES

AACC. 1995. Approved Methods of the American Association of Cereal Chemists. 9th ed. American Assoc. Cereal Chemists, St. Paul, MN.

Baker, R.J. 1977. Inheritance of kernel hardness in spring wheat. Crop Sci. 17:960–962.

Barlow, K.K.K., M.S. Buttrose, D.H. Simmonds, and M. Vesk. 1973. The nature of the starch-protein interface in wheat endosperm. Cereal Chem. 50:443–454.

Bayles, B.B., and J.A. Clark. 1954. Classification of wheat varieties grown in the United States in 1949. USDA Tech. Bull. 1083.

Bergman, C.J., D.G. Gualberto, K.G. Campbell, M.E. Sorrels, and P.L. Finney. 1998. Genotype and environment effects on wheat quality traits in a population derived from a soft by hard cross. Cereal Chem. 75:729–737.

Bettge, A.D., C.F. Morris, and G.A. Greenblatt. 1995. Assessing genotypic softness in single wheat kernels using starch granule-associated friabilin as a biochemical marker. Euphytica 86:65–72.

Blochet, J.E., C. Chevalier, E. Forest, E. Pebay-Peyroula, M.F. Gautier, P. Joudrier, M. Pezolet, and D. Marion. 1993. Complete amino acid sequence of puroindoline, a new basic and cysteine-rich protein with a unique tryptophan-rich domain, isolated from wheat endosperm by Triton X-114 phase partitioning. FEBS Lett. 329:336–340.

Campbell, K.G., C.J. Bergman, D.G. Gualberto, J.A. Anderson, M.J. Giroux, G. Hareland, R.G. Fulcher, M.E. Sorrels, and P.L. Finney. 1999. Quantitative Trait Loci associated with kernel traits in a Soft × Hard wheat cross. Crop Sci. 39:1184–1195.

Crosbie, G.B. 1989. Soft wheat: View from Australia. Cereal Foods World 34:678–681.

Gautier, M.F., M.E. Aleman, A. Guirao, D. Marion, and P. Joudrier. 1994. Triticum aestivum puroindolines, two basic cysteine-rich seed proteins: cDNA analysis and developmental gene expression. Plant Mol. Biol. 25:43–57.

Giroux, M.J., and C.F. Morris. 1997. A glycine to serine change in puroindoline b is associated with wheat grain hardness and low levels of starch-surface friabilin. Theor. Appl. Genet. 95:857–864.

Giroux, M.J., and C.F. Morris. 1998. Wheat grain hardness results from highly conserved mutations in the friabilin components puroindoline a and b. Proc. Natl. Acad. Sci. USA 95:6262–6266.

Greenblatt, G.A., A.D. Bettge, and C.F. Morris. 1995. The relationship between endosperm texture, and the occurrence of friabilin and bound polar lipids on wheat starch. Cereal Chem. 72:172–176.

Greenwell, P., and J.D. Schofield. 1986. A starch granule protein associated with endosperm softness in wheat. Cereal Chem. 63: 379–380.

Haro, E.S. 1990. Heading date and associated plant responses in near-isogenic lines of the soft white winter wheat Nugaines. Ph.D. diss. (Diss. Abstr. AAT 9131064). Washington State Univ., Pullman, WA.

Haro, E.S., and R.E. Allan. 1997. Effects of heading date on agronomic performance of winter wheat isolines. Crop Sci. 37:346–351.

Jolly, C.J., S. Rahman, A.A. Kortt, and T.J.V. Higgins. 1993. Characterization of the wheat Mr-15000 'grain-softness protein' and analysis of the relationship between its accumulation in the whole seed and grain softness. Theor. Appl. Genet. 86:589–597.

Law, C.N., C.F. Young, J.W.S. Brown, J.W. Snape, and J.W. Worland. 1978. The study of grain protein control in wheat using whole chromosome substitution lines. p. 483–502. *In* Seed protein improvement by nuclear techniques. International Atomic Energy Agency, Vienna, Austria.

Lillemo, M., and C.F. Morris. 2000. A leucine to proline mutation in puroindoline b is frequently present in hard wheats from Northern Europe. Theor. Appl. Genet. 100:1100–1107.

Martin, C.R., R. Rouser, and D.L. Brabec. 1993. Development of a single-kernel wheat characterization system. Trans. ASAE 36(5): 1399–1404.

Mattern, P.J., R. Morris, J.W. Schmidt, and V.A. Johnson. 1973. Locations of genes for kernel properties in the wheat variety 'Cheyenne' using chromosome substitution lines. p. 703–707. *In* E.R. Sears and L.M.S. Sears (ed.) Proc. Int. Wheat Genet. Symp., 4th, Columbia, MO. 6–11 Aug. 1973. University of Missouri, Columbia, MO.

Morris, C.F., M. Lillemo, M.C. Simeone, M.J. Giroux, S.L. Babb, and K.K. Kidwell. 2001. Prevalence of puroindoline grain hardness genotypes among historically significant North American spring and winter wheats. Crop Sci. 41:218–228 (this issue).

Morris, C.F., and R.E. Allan. 2001. Registration of hard and soft near-isogenic lines of hexaploid wheat genetic stocks. Crop Sci. 41(3) in press.

Morris, C.F., G.A. Greenblatt, A.D. Bettge, and I. Malkawi. 1994. Isolation and characterization of multiple forms of friabilin. J. Cereal Sci. 20:167–174.

Morris, C.F., V.L. DeMacon, and M.J. Giroux. 1999. Wheat grain hardness among chromosome 5D homozygous recombinant substitution lines using different methods of measurement. Cereal Chem. 76:249–254.

Morris, C.F. 1992. Impact of blending hard and soft white wheats on milling and baking quality. Cereal Foods World 37:643–648.

Morris, C.F., and S.P. Rose. 1996. Chapter 1. Wheat. p. 3–54. *In* R.J. Henry and P.S. Kettlewell (ed.) Cereal grain quality. Chapman Hall, London.

Nagao, S., S. Ishibashi, S. Imai, T. Sato, T. Kanbe, Y. Kaneko, and H. Otsubo. 1977. Quality characteristics of soft wheats and their utilization in Japan. II. Evaluation of wheats from the United States, Australia, France and Japan. Cereal Chem. 54:198–204.

Norris, K.H., W.R. Hruschka, M.M. Bean, and D.C. Slaughter. 1989. A definition of wheat hardness using Near Infrared Reflectance spectroscopy. Cereal Foods World 34:696–705.

Oda, M., Y. Yasuda, S. Okazaki, Y. Yamauchi, and Y. Yokoyama. 1980. A method of flour quality assessment for Japanese noodles. Cereal Chem. 57:253–254.

Pomeranz, Y., and P.C. Williams. 1990. Wheat hardness: Its genetic, structural, and biochemical background, measurement, and significance. p. 471–548. *In* Y. Pomeranz (ed.) Advances in cereal science and technology, Vol. X. Am. Assoc. Cereal Chemists, St. Paul, MN.

Rogers, D.E., R.C. Hoseney, G.L. Lookhart, S.P. Curran, W.D.A. Lin, and R.G. Sears. 1993. Milling and cookie baking quality of near-isogenic lines of wheat differing in kernel hardness. Cereal Chem. 70:183–187.

SAS. 1989. SAS/STAT User's guide. 4th ed. Version 6. SAS Institute Inc., Cary, NC.

Simmonds, D.H. 1974. Chemical basis for hardness and vitreosity in the wheat kernel. Bakers Dig. 48:16–29, 63.

Symes, K.J. 1969. Influence of a gene causing hardness on the milling and baking quality of two wheats. Aust. J. Agric. Res. 20:971–979.

Symes, K.J. 1965. The inheritance of grain hardness in wheat as measured by the particle-size index. Aust. J. Agric. Res. 16:113–123.

Williams, P.C. 1979. Screening wheat for protein and hardness by near infrared reflectance spectroscopy. Cereal Chem. 56:169–172.